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Recurring infection by crayfish plague pathogen only marginally affects survival and growth of marbled crayfish

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Abstract

Invasive alien crayfish threaten the diversity of freshwater ecosystems and native crayfish fauna. In Europe, this is largely due to transmission of the crayfish plague to susceptible native crayfish. Many invasive species tolerate crayfish plague, but the infection still has the potential to reduce the fitness of a tolerant host due to energy trade-offs between immune response maintenance and life-history traits, such as growth and reproduction. In combination with other unfavourable conditions, such a response could alter further invasion success of an otherwise successful crayfish invader. We examined whether repeated infection with one of the most virulent haplogroups of crayfish plague agent (*Aphanomyces astaci*) affects growth or survival of the juvenile marbled crayfish (*Procambarus virginalis*). Juveniles were exposed to i) two levels of pathogen concentrations, and ii) two different feeding regimes under the higher pathogen concentration. In all performed trials, repeated infection reduced growth rates, while the combination of recurring infection and food limitation significantly increased mortality. The average energy cost of the immune response was estimated at 12.07 J/day for individuals weighing 0.3 grams. Since infections were frequent

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and pathogen concentrations high, results suggest that marbled crayfish is resistant to *A. astaci* pathogen and its survival is only affected by adding the stress of food limitation. The survival of almost half of the individuals exposed to high pathogen loads and extreme food limitation indicates that chronic infection by crayfish plague is unlikely to be an important factor impeding invasion success of the marbled crayfish, even under harsh conditions. Our results add to the growing body of evidence that marbled crayfish has potential to become one of the most successful freshwater invaders.

Keywords
food limitation, freshwater, immunity cost, infection, invasive species, trade-off

Introduction

Invasive alien species drive biodiversity loss and impair ecosystem services worldwide (Pyšek and Richardson 2010), partly because they carry pathogens to which native species lack resistance. In freshwater ecosystems, crayfish directly affect ecosystem structure and diversity (Dorn and Wojdak 2004). Many native crayfish, particularly in Europe, are on the brink of extinction due to introductions of invasive crayfish and their pathogens (Jussila et al. 2021; Theissinger et al. 2021). Invasive crayfish imported from North America into Europe introduced a novel and lethal disease, the crayfish plague, caused by the oomycete *Aphanomyces astaci* Schikora, 1906 (Jussila et al. 2021; Theissinger et al. 2021). The introduction and transmission of pathogen *A. astaci* is the main reason why native crayfish were displaced by invaders, and crayfish plague is solely responsible for decimation of numerous native European crayfish populations (Martín-Torrijos et al. 2019; Jussila et al. 2021; Theissinger et al. 2021).

North American crayfish species tolerate the crayfish plague due to their rapid immune reaction to the pathogen. The fast activation of the prophenoloxidase (ProPO) system encapsulates the pathogen's hyphae in melanin (Cerenius et al. 2003). However, even in North American crayfish, stressful conditions (i.e. chronic infections, unfavourable environmental conditions) may increase their susceptibility to pathogens (Chinchio et al. 2020) or negatively affect their fitness, growth and development (cf. Francesconi et al. 2021). Chronic infection requires continuous mounting of an immune response even in a tolerant species, thus incurring energy costs. In other species, covering these costs requires energy redistribution that results in trade-offs with life-history traits, such as growth and reproduction (e.g. Lochmiller and Deerenberg 2000; Martin et al. 2003, 2008; Rantala and Roff 2005; Lee 2006; van Der Most et al. 2011; Brown and Shine 2014; Schwenke et al. 2016; Kirschman et al. 2017; Körner et al. 2017; Miyashita et al. 2019). These trade-offs are most apparent under stressful conditions (i.e. limited resources or environmental/physiological stress; Kirschman et al. 2017; Rumschlag and Boone 2020) and may cause death (Garner et al. 2009) and ultimately adversely affect host population dynamics (Boots and Norman 2000; Boots et al. 2003; Campbell et al. 2019). Therefore, while chronic exposure to *A. astaci* may be sublethal in a resistant invasive crayfish species, it can cause mortality if individuals are under additional stress (Aydin et al. 2014). Such circumstances may lead to

population crashes even in tolerant invaders (e.g. as in signal crayfish; Edsman et al. 2015), alter their further invasion success, and even mitigate their negative effects on the ecosystem (Fincham et al. 2019; Chinchio et al. 2020).

We analyse whether chronic exposure to *A. astaci* may lead to significant sublethal or lethal effects in the marbled crayfish (*Procambarus virginalis* Lyko 2017, Cambaridae), a high-risk crayfish invader in Europe (Chucholl 2016; Kouba et al. 2021; Vogt 2021), listed amongst the Invasive Alien Species of Union concern (EU Regulation No. 1143/2014). Marbled crayfish is the only obligatory parthenogenetic freshwater crayfish (Scholtz et al. 2003) producing a large number of genetically identical offspring (Martin et al. 2007; Vogt et al. 2008), which makes it an ideal model organism. Although genealogically most closely related to North American species *Procambarus fallax* (Hagen, 1870), it has no known native habitat (Lyko 2017; Hossain et al. 2018). Its occurrence in Europe is significantly increasing due to its popularity in pet-trade (Chucholl 2016), as are contacts with other crayfish invaders that are known *A. astaci* carriers (cf. Linzmaier et al. 2020). Marbled crayfish can carry *A. astaci* haplogroup D, associated with another invader, *Procambarus clarkii* (Girard, 1852) (Keller et al. 2014; Mrugała et al. 2015). It is resistant to low concentrations of a highly virulent *A. astaci* haplogroup B, associated with invasive signal crayfish, *Pacifastacus leniusculus* (Dana, 1852) (Francesconi et al. 2021). However, the impact of chronic infection by crayfish plague and its combined effect with other stressors on survival and life history of marbled crayfish have not been explored yet, even though they could potentially affect its future expansion.

Methods

Experimental animals

Juvenile marbled crayfish were taken from an adult female transferred from Šoderica Lake in northern Croatia (46°14'20.9"N, 16°54'33.6"E) to the laboratory of the Department of Biology at the Faculty of Science in Zagreb. Adult marbled crayfish were kept in the laboratory for five months prior to oviposition, when juveniles for the experiments were obtained. For marbled crayfish keeping and research, we obtained a licence from the responsible Ministry of Environment and Energy, Croatia (Licence number: UP/I-612-07/19-43/01; 517-05-1-1-20-6), in accordance with the EU Regulation on Invasive Alien Species (EU Regulation No. 1143/2014).

Juvenile marbled crayfish were used in all experiments, since they moult often, allowing faster assessment of effects of exposure to *A. astaci* pathogen on growth and since juvenile growth is linear (McLay and van den Brink 2016).

Housing of experimental animals

Juvenile marbled crayfish were kept individually in 100 ml of aerated tap water in 250 ml plastic containers with a diameter of 90 mm. Containers were placed in cooled

incubators (ST 6 COMF, POL-EKO-APARATURA, Poland) 16–18 days before the start of the experiments to allow the juveniles to adapt to housing conditions (temperature regime 20 °C, photoperiod 12:12 light:dark). To avoid cross-contamination, each experimental group (described below) was housed in a separate incubator. The selected temperature results in the lowest mortality in the laboratory (Vogt et al. 2004). An aquarium pump (JK-AP9500, JK ANIMALS, Czech Republic) was placed between the containers and all containers were covered with a plastic lid to prevent water evaporation and escape of the crayfish. The crayfish were fed one pellet of JBL NovoPrawn food for shrimps with frequency depending on the feeding regime, and the water in all the containers was changed twice a week.

Aphanomyces astaci zoospore production

Haplogroup B strain of *A. astaci*, associated with the signal crayfish (Francesconi et al. 2021), was selected for the experiment because of its potential horizontal transmission from the signal crayfish to marbled crayfish. Šoderica Lake is close (ca. 450 m) to the Drava River, which contains signal crayfish infected with *A. astaci* (Maguire et al. 2016) and contact between the two species is expected to occur (Dobrović et al. 2021). Additionally, the signal crayfish is the most widespread invasive crayfish species in Europe, present in over 29 EU countries and in EU candidate countries (Dragičević et al. 2020), making transmission of this highly virulent haplogroup to the marbled crayfish likely.

A. astaci PEC8 isolate (haplogroup B) was obtained from Prof. Frédéric Grandjean from University of Poitiers, France. The isolate was grown on PG1 agar (Unestam 1965) with addition of antibiotics - oxolinic acid (SIGMA 0-00877) and ampicillin (SIGMA A 9518) at a concentration of 10 mg/l, to prevent bacterial growth. *A. astaci* zoospore production was induced using a protocol, based on Makkonen et al. (2012). A piece of PG1 agar (4 × 4 mm) containing *A. astaci* hyphae was used to inoculate 45 ml of liquid PG1 medium (Unestam 1965) with antibiotics in 12 replicates. The replicates were incubated at 18 °C for four days, after which the hyphae were cut into small fragments and agar was removed. Total hyphae biomass was divided into four portions, each portion was transferred into 50 ml of fresh liquid PG1 medium with antibiotics and grown at 18 °C for three days. Liquid PG1 medium was then removed and sporulation was induced by washing the hyphae in water from the stream Vrapčak in Gornje Vrapče, Zagreb (45°50'36.3"N, 15°53'44.8"E). Hyphae were washed four times with 200 ml of autoclaved natural water with mild linear shaking for 45 minutes at 18 °C, and were left in the shaker for 20 hours in the last (fourth) wash. Hyphae were then removed and zoospore concentration in the suspension was counted using a Thoma counting chamber. Crayfish infection was performed immediately after the zoospore production by adding zoospores to crayfish containers. Experimental zoospore concentrations (described below) in 100 ml of water were obtained by diluting the produced zoospore suspension with aerated tap water.

Experimental design

Prior to the infection experiment, virulence of the PEC8 isolate and sublethal load was tested in a pilot experiment to determine two sublethal concentrations of *A. astaci* zoospores and frequency of the repeated infections (Vukelić 2021). Infections were set to be repeated every two weeks at concentrations of 7500 zoospores/ml and 15000 zoospores/ml in Experiment 1, and 15000 zoospores/ml in Experiment 2 (described below). Dosages were higher than in other studies involving infection trials on marbled crayfish (i.e. Francesconi et al. 2021), but still within a range used for tolerant crayfish invaders (i.e. 10000 zoospores/ml in signal crayfish infection trials; Aydin et al. 2014) and considered realistic in the case of acute infection in the wild (Aydin et al. 2014).

During the experiments, crayfish from all experimental groups (control and infected groups) were photographed and weighed i) directly before each infection, ii) two weeks after the last infection (Experiment 1 and 2), and iii) six weeks after the last infection (Experiment 1). Due to the small size of the juveniles, their total length (TL; from the top of the rostrum to the end of the telson, in mm) was measured from photographs using the image processing programme, ImageJ (Schneider et al. 2012; http://rsbweb.nih.gov/ij/). Each crayfish was measured three times and the mean value represented TL. Before weighing, each crayfish was carefully dried on filter paper to remove excess water and weighed using a digital scale (accuracy: ± 0.0001 g). During the experiment, we also recorded mortality, disease symptoms (melanisation of the cuticle) and moulting of the crayfish. At the end of the experiments, surviving crayfish were euthanised by a rapid cut of the nerve cord through the thorax to the abdomen, in accordance with available guidelines for humane killing of crayfish (Conte et al. 2021). All specimens were stored in 96% ethanol for detection of *A. astaci* DNA from crayfish cuticle as described in Suppl. material 1: S1, while in surviving crayfish from Experiment 2, hepatopancreas was additionally dissected for gene expression analysis (described below).

Experiment 1: Repeated exposure of juvenile marbled crayfish to different *A. astaci* zoospore concentrations

A total of 55 juveniles were used in this experiment. For the trial, juvenile marbled crayfish were randomly divided into three experimental groups: 1) control group (15 individuals, non-infected), 2) group 7500 (20 individuals infected with 7500 zoospores/ml), and 3) group 15000 (20 individuals infected with 15000 zoospores/ml). The infection experiment was conducted for 18 weeks, with *A. astaci* infection performed every other week, six infections in total. In one case, the third infection was performed four weeks after the second infection due to unsuccessful *A. astaci* zoospore production at the time. Surviving crayfish were euthanised six weeks after the last infection and the cuticle of all crayfish was tested for presence of *A. astaci* (those that died during the experiment and those that survived until the end of the experiment; crayfish plague detection described in Suppl. material 1: S1).

Experiment 2: Repeated exposure of juvenile marbled crayfish to *A. astaci* under differing feeding regimes

A total of 60 individuals from a separate batch were used to test the interaction of chronic exposure to *A. astaci* with food availability. While food availability is unlikely to be a limiting factor in nature because crayfish are omnivores (Holdich 2002), it was used as a proxy for density-dependent effects. Juvenile crayfish form social dominance hierarchies (Issa et al. 1999; Sato and Nagayama 2012) and dominant individuals have increased access to food (Herberholz et al. 2007). Increasing population density is thus expected to limit resource availability, increase competition intensity (Capelli and Hamilton 1984), and induce stress.

In this experiment, crayfish were adapted to laboratory conditions for 16 days during which all individuals were fed twice a week. Then, four experimental groups of 15 individuals were used: 1) control - fed five times a week, non-infected, 2) control, food-restricted - fed once a week, non-infected, 3) infected - fed five times a week, and 4) infected, food-restricted - fed once a week. This experiment was conducted for 12 weeks, also with *A. astaci* infection intervals every other week (five in total).

Surviving crayfish were euthanised two weeks after the last infection and cuticle samples of all crayfish were tested for *A. astaci*, as in Experiment 1 (crayfish plague detection described in Suppl. material 1: S1). Additionally, euthanised crayfish were dissected and the hepatopancreas from each was carefully removed and stored in an RNA stabilising agent (RNA later; Sigma Aldrich, MO, USA) at -80 °C until RNA extraction for gene expression analysis. Five random individuals from each group were analysed for changes in expression of two genes related to crayfish innate immunity (prophenoloxidase - ProPO and CCAAT/enhancer-binding protein beta - C/EBP-β), and central metabolic pathways of glycolysis and citrate cycle (citrate synthase - CS and glyceraldehyde 3-phosphate dehydrogenase - GAPDH), selected based on Boštjančić et al. (2021) and Zheng et al. (2021). Total RNA was isolated with the RNeasy Lipid Tissue Mini Kit (Qiagen, Germany). RNA quality was checked on the NanoVue Spectrophotometer, and RNA quantity with the QuantiFluor RNA System on the Quantus platform (Promega, USA). For the cDNA synthesis, 1 μg of total RNA was reversely transcribed with the iScript Select cDNA Synthesis Kit (Bio-Rad, USA) and Oligo(dT)15 primer. For the quantitative reverse transcription PCR (RT-qPCR), target loci were amplified in 10 μl reactions with iTaq Universal SYBR Green Supermix (Bio-Rad, USA), with 1 μl of input cDNA template on the CFX Opus 96 Real-Time PCR (Bio-Rad, USA). All samples were run in duplicates with the standard deviation of cycle threshold values < 0.5. Primer pairs for all target genes and endogenous control (elongation factor 1-α) can be found in Suppl. material 1: Table S2.1. The difference in the gene expression values between the target and control samples was expressed according to the delta-delta Ct method ($2^{-\Delta\Delta CT}$; Livak and Schmittgen 2001).

Estimating energy cost of the infection

We estimated energy cost of immunity response to chronic infection based on the differences in weight of non-infected and infected food-restricted groups in Experiment

2. Neither of the food-restricted groups grew appreciably in length, so differences in weight change between the two groups stem from differences in energy reserve dynamic: if size is the same, lighter individuals have smaller energy reserves. The reduction in energy reserves can be explained either by smaller energy input (e.g. reduction in food intake due to infection) or a higher basal metabolic rate related to the infection. Food intake was equal for both groups because all food had been eaten, so reduction in energy reserves must have been caused by increased metabolism due to the infection. Therefore, the energy content corresponding to the difference in weight represents the cost of the infection. In crayfish, changes in energy reserves are primarily reflected in change of hepatosomatic index, i.e. hepatopancreas weight (Jussila and Mannonen 1997; Sacristán et al. 2017). Energy content of hepatopancreas (E; kJ/g) of marbled crayfish was estimated using the correlation with moisture content of hepatopancreas (M; %) as suggested by Jussila and Mannonen (1997), using the regression for *Astacus astacus* (Linnaeus, 1758), for pooled sexes:

$$E = -0.336M + 54.189. \quad (1)$$

Average moisture content of hepatopancreas of marbled crayfish from our sampling site was reported to be 62% (Žižak 2015) and, therefore, this value was used to calculate energy reserve content. Finally, the difference in weight change between the groups was multiplied by the estimated energy content and divided by the duration of experiment to obtain cost of immunity response per day.

Statistical analysis

The effects of repeated infections using two *A. astaci* concentrations and interactive effects of repeated infections and food availability were examined through three major endpoints: 1) total growth (i.e. weight/length gain throughout the total duration of both experiments), 2) rate of growth (i.e. weight/length increment over time, in each experimental week, presented in Suppl. material 1: S3–S5), and 3) mortality (measured through the whole experimental period). In addition, the changes in immune and metabolic gene expression and metabolism were analysed in Experiment 2. Statistical analyses and graphical representation of results were performed in the R statistical environment (R version 3.6.3, R Studio 1.2.5033). The '*base*' and '*pastecs*' packages (Grosjean and Ibanez 2018; R Core Team 2020) were used for descriptive statistics and basic homoscedasticity (Levene test), normality (Shapiro-Wilks, QQ-plot) and extreme value tests, while the '*ggplot2*' package was used for graphical display.

Total growth

Robust ANOVAs based on trimmed means (20% of trimming level; Wilcox 2012; Mair and Wilcox 2020; '*WRS2*' package in R) were used to test (i) the effects of *A. astaci* infection (Experiment 1; one-way factorial design; three levels), and (ii) the joint effects of *A. astaci* infection and food availability (Experiment 2; two-way

factorial design; 2 × 2 levels) on total weight (length) gain. Total weight (length) gain was calculated as the difference between the final weight (length) at the end of the experiment and the initial weight (length) at the beginning of the experiment. Robust ANOVAs were used instead of classical ANOVAs to overcome problems associated with deviations from homoscedasticity and to reduce the influence of outliers observed in the data. Post-hoc tests were also performed in the robust '*WRS2*' environment (Mair and Wilcox 2020), while p-values were adjusted for multiple testing using the Benjamini-Hochberg (BH) method. The '*multcompView*' package (Graves et al. 2019) was used to convert the vector of p-values to a character-based display in which common characters denote levels or groups that are not significantly different.

Rate of growth

Growth rate analysis was performed to analyse questions such as: (a) what average growth trajectory best describes the rate of growth over time for all crayfish, (b) what is the variability in growth rates across crayfish, and finally, (c) does *A. astaci* infection and food availability explain variability in growth rates? Growth rate analysis, unlike total growth analysis, is based on complete longitudinal measurements of weight (and/or length) rather than just initial and final values. As the measurements at specific time points are clustered (nested) within individual crayfish, they imply a hierarchical structure of the data and intra-individual correlation. Therefore, the multilevel modelling (MLM) technique was used for data analysis (Peugh 2010; Monsalves et al. 2020). A detailed specification of all data levels, model variables and model equations can be found in Suppl. material 1: S3.

Mortality

Fisher's exact tests were used to determine the association between exposure to *A. astaci* and mortality in both experiments. Pairwise (post-hoc) Fisher test from the '*rstatix*' package in R (Kassambara 2020) with the Holm method of adjusting p-values was used for multiple comparisons of groups in Experiment 2. Kaplan-Meier estimates (curves) of survival probability and comparison of survival times between groups by the log-rank test were performed using the '*survival*' and '*survminer*' packages in R (Kassambara et al. 2020; Therneau 2022).

Gene expression

The Kruskal-Wallis test was used to determine whether the expression of immune and metabolic genes differed significantly between the groups in Experiment 2. Dunn's test from the '*FSA*' package in R (Ogle et al. 2021) was used for multiple pairwise comparisons of the groups. In the Dunn test, p-values were adjusted according to

the Holm method. To further investigate the relationships between gene expression variables, the '*FactoMineR*' and '*factoextra*' packages in R (Le et al. 2008; Kassambara and Mundt 2020) were used for principal component analysis (PCA) of the gene expression dataset.

Data availability

Data are permanently deposited in an open repository (Dryad: https://doi.org/10.5061/dryad.3xsj3txkp).

Results

Infection success

In both experiments, all crayfish that died (marked in red colour in Fig. 1) tested *A. astaci* positive, while all control animals tested to be pathogen-free (Fig. 1). Overall, the proportion of *A. astaci* positive individuals was higher in Experiment 2 (*A. astaci* detection two weeks since the last infection; Fig. 1B) than in Experiment 1 (*A. astaci* detection six weeks since the last infection; Fig. 1A).

Experiment 1: Repeated exposure of juvenile marbled crayfish to different *A. astaci* zoospore concentrations

Total growth

Repeated exposure to the crayfish plague pathogen (Fig. 2A) significantly reduced total weight gain ($F(2,17.06) = 10.04$, $p = 0.001$, effect size $\xi = 0.84$, $CI(\xi) = [0.64, 1.11]$; Suppl. material 1: Table S4.1C). Both exposed groups weighed less than the control group, but did not differ from each other (Fig. 2A; post-hoc tests in Suppl. material 1: Table S4.1E). Total length gain (Suppl. material 1: Fig. S4.1A) was not significantly different between treatments ($F(2,17.19) = 1.86$, $p = 0.185$; Suppl. material 1: Table S4.1D).

Rate of growth

Due to the complexity of the MLM model, a detailed description of the results on individual and group growth rates is given in Suppl. material 1: S3.2, S4, Figs S4.2, S4.3, Tables S4.2, S4.3). Here, we provide only a summary of the main results (fixed effects). *A. astaci* infection significantly reduced growth rates: the growth rate of weight in the control group (12.7 mg/wk) was significantly higher

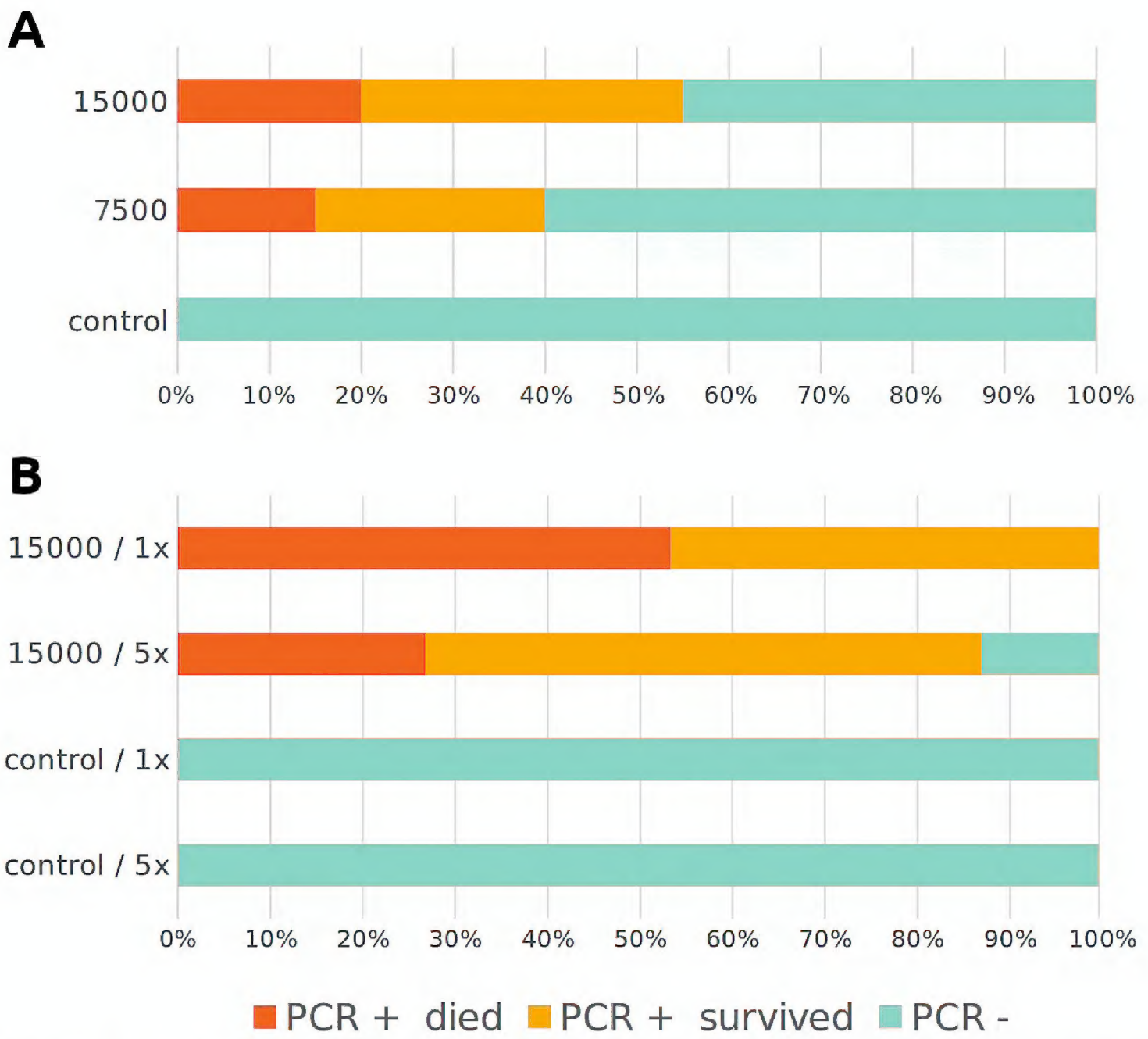

Figure 1. Detection of *A. astaci* in the cuticle of marbled crayfish in **A** Experiment 1 and **B** Experiment 2. In Experiment 1, surviving crayfish were euthanised six weeks after the last infection; in Experiment 2, they were euthanised after two weeks. PCR+ = *A. astaci* detected, PCR - = *A. astaci* not detected.

than the growth rates in both *A. astaci* infected groups (10.2 mg/wk and 9.6mg/wk; Suppl. material 1: Table S4.2D), while growth rate of length was significantly reduced only under higher *A. astaci* concentration (Suppl. material 1: Table S4.3D). Weight and length increased significantly over time in all groups.

Mortality

The effect of exposure to pathogens on mortality was not significant ($p = 0.26$, Fisher's exact test). A similar result, i.e. insignificant effect of infection, was also inferred from the overlapping confidence intervals of the Kaplan-Meier survival curves (Fig. 2C) and from the comparison of survival times between the groups using the log-rank test ($\chi^2(2) = 3.2$, $p = 0.20$).

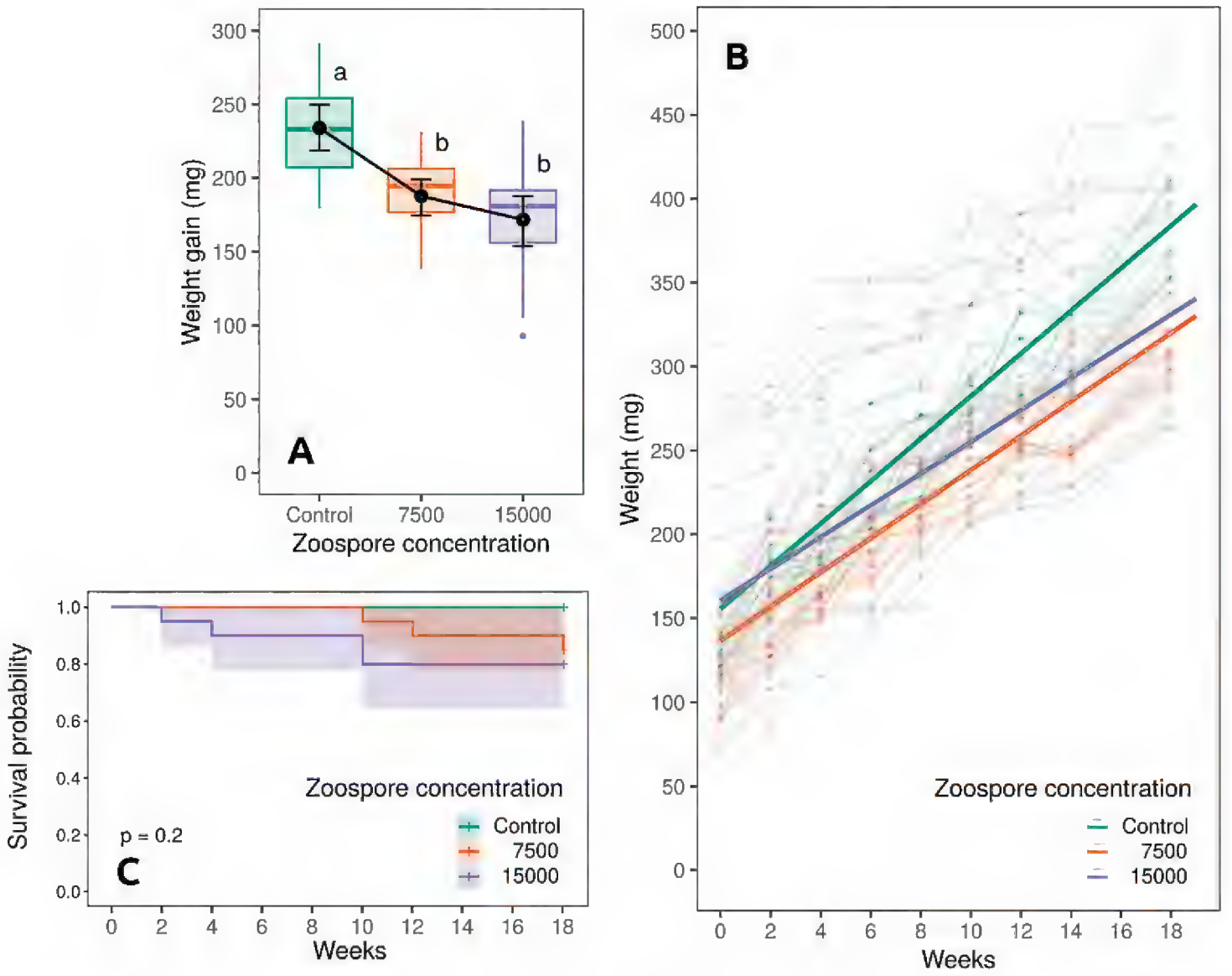

Figure 2. Effects of repeated infection of marbled crayfish juveniles using two *A. astaci* zoospore concentrations (7500 and 15000 zoospore/ml) on **A** total weight gain (total growth) **B** rate of weight increment (rate of growth) and **C** survival probability of individuals. Different letters in panel A denote significant differences, error bars represent 95% confidence intervals (CIs) around the mean. Results for total length gain and rates of length increment are presented in Suppl. material 1: S4.

Experiment 2: Repeated exposure of juvenile marbled crayfish to *A. astaci* under differing feeding regimes

Total growth

Food availability (i.e. different feeding regimes) significantly influenced crayfish growth (Fig. 3A). There was a significant effect of food ($Q = 652.22$, $p = 0.001$), *A. astaci* infection ($Q = 44.55$, $p = 0.001$), and their interaction ($Q = 11.92$, $p = 0.003$) on the total weight gain during the experiment (Suppl. material 1: Table S5.1C). Pairwise post-hoc comparisons revealed significant differences in weight gain between all groups (Suppl. material 1: Table S5.1D). Food-restricted groups gained significantly less weight - both in the control ($\hat{\psi} = 279.11$, $p < 10^{-5}$) and the infected groups ($\hat{\psi} = 212.62$, $p < 10^{-5}$). Weight gain of the control food-restricted group was approximately 90% lower than in the control group fed five times

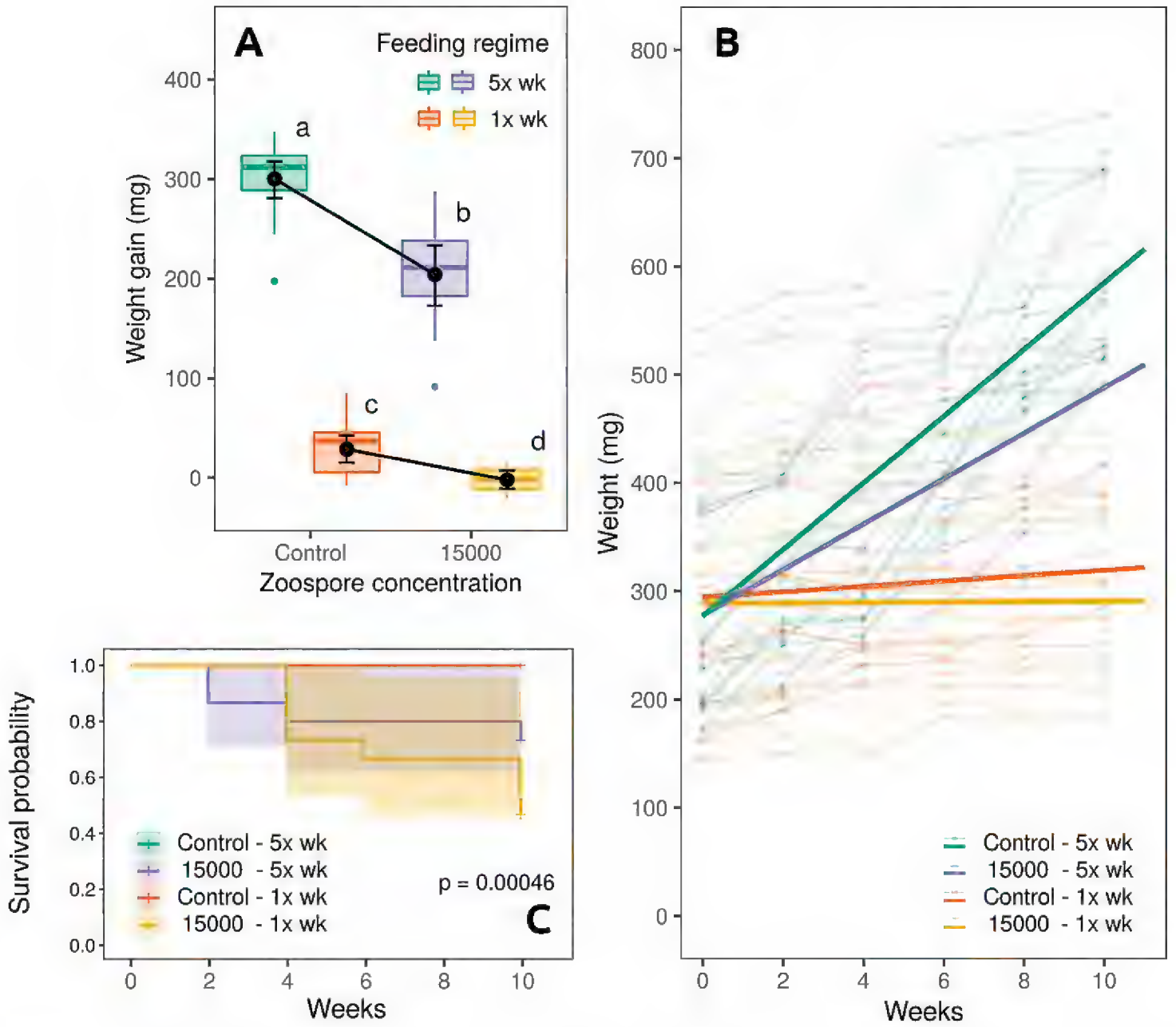

Figure 3. Effects of repeated infection of marbled crayfish juveniles with 15000 zoospore/ml of *A. astaci* under two feeding regimes (once a week and five times a week) on **A** total weight gain (total growth) **B** rate of weight increment (rate of growth) and **C** survival probability of individuals. Different letters in panel A denote significant differences, error bars represent 95% confidence intervals (CIs) around the mean. Results for total length gain and rates of length increment are presented in Suppl. material 1: S5.

a week (Suppl. material 1: Table S5.1A). Total weight gain was lower when crayfish were infected, compared to non-infected control groups (feeding regime five times per week: $\hat{\psi} = 97.50$, p = 0.0002; feeding regime once a week: $\hat{\psi} = 31.01$, p = 0.014; Suppl. material 1: Table S5.1D). In general, the effect of food on weight gain ($\xi = 0.93$, CI(ξ) = [0.89; 1]) was stronger than the effect of *A. astaci* infection (ξ = 0.21, CI(ξ) = [0.03; 0.56]). Furthermore, the effect of infection was stronger at high food than food-restricted regime due to significant interactions between food and weight (Suppl. material 1: Table S5.1C). Additional inferential analysis of length as a response variable leads to similar conclusions, but without significant differences in total length gain between infected and non-infected groups under food restriction (p = 0.07; Suppl. material 1: Fig. S5.1A, Table S5.1B, E, F).

Rate of growth

A detailed analysis of the results of the MLM model can be found in Suppl. material S3.3 and S5, Figs S5.2, S5.3, Tables S5.2, S5.3); here, a summary of the main results (fixed effects) is given. Both food restriction and infection with *A. astaci*, as well as their interactive effect, significantly reduced growth rate of weight (and length). The average growth rate of uninfected crayfish fed five times per week was 30.8 mg/wk, compared with an average growth rate of 21.0 mg/wk for the infected group under the same feeding regime, and an average growth rate of 2.5 mg/wk for the food-restricted control group. The average growth rate of the infected crayfish with food restriction was only 0.2 mg/wk. The infection-induced decrease in growth rate was greater when food was abundant than when food was restricted.

Mortality

Food limitation and *A. astaci* infection increased individual mortality. Mortality differed significantly amongst groups in Experiment 2 ($p < 10^{-3}$, Fisher's exact test), with the highest mortality occurring in the infected group fed once a week (8 deaths, i.e. 53% in 10 weeks; Fig. 3C). The statistically significant difference in mortality in pairwise comparisons was obtained only between this group and (i) the control (non-infected) group fed once per week ($p = 0.013$) and (ii) the control group fed five times per week ($p = 0.013$). The difference in mortality rate between the infected and non-infected groups fed five times a week was not significant ($p = 0.398$). Consistent with the result of Fisher's test, survival times estimated by Kaplan-Meier method (Fig. 3C) were significantly different between groups according to the log-rank test ($\chi^2(3) = 17.9$, $p < 10^{-3}$).

Expression of immune and metabolic genes

Repeated exposure to *A. astaci* and different food availability significantly affected the expression of the metabolic genes CS ($H(3) = 15.34$, $p = 0.002$) and GAPDH ($H(3) = 9.72$, $p = 0.021$), and the immune gene C/EBP-β ($H(3) = 14.54$, $p = 0.002$), with no significant effects on ProPO expression (Fig. 4A; Suppl. material 1: Table S5.4A, C, E, G). The expression of metabolic genes was reduced in groups fed once a week, but the significantly lower mean rank was confirmed by post-hoc analysis only for the infected group (CS: $p = 0.01$; GAPDH: $p = 0.038$; Suppl. material 1: Table S5.4B, D). Conversely, the expression of the immune gene C/EBP-β was elevated in the groups fed once a week, but again, a significantly higher mean rank was confirmed by post-hoc analysis only for the infected group ($p = 0.007$; Suppl. material 1: Table S5.4F).

Relationships amongst gene expression variables were further analysed using the principal component analysis. Two principal components captured most of the variability in the dataset (87.6%, Fig. 4B), justifying the reduction of the complexity of the data analysis from four dimensions to two dimensions. The first principal component

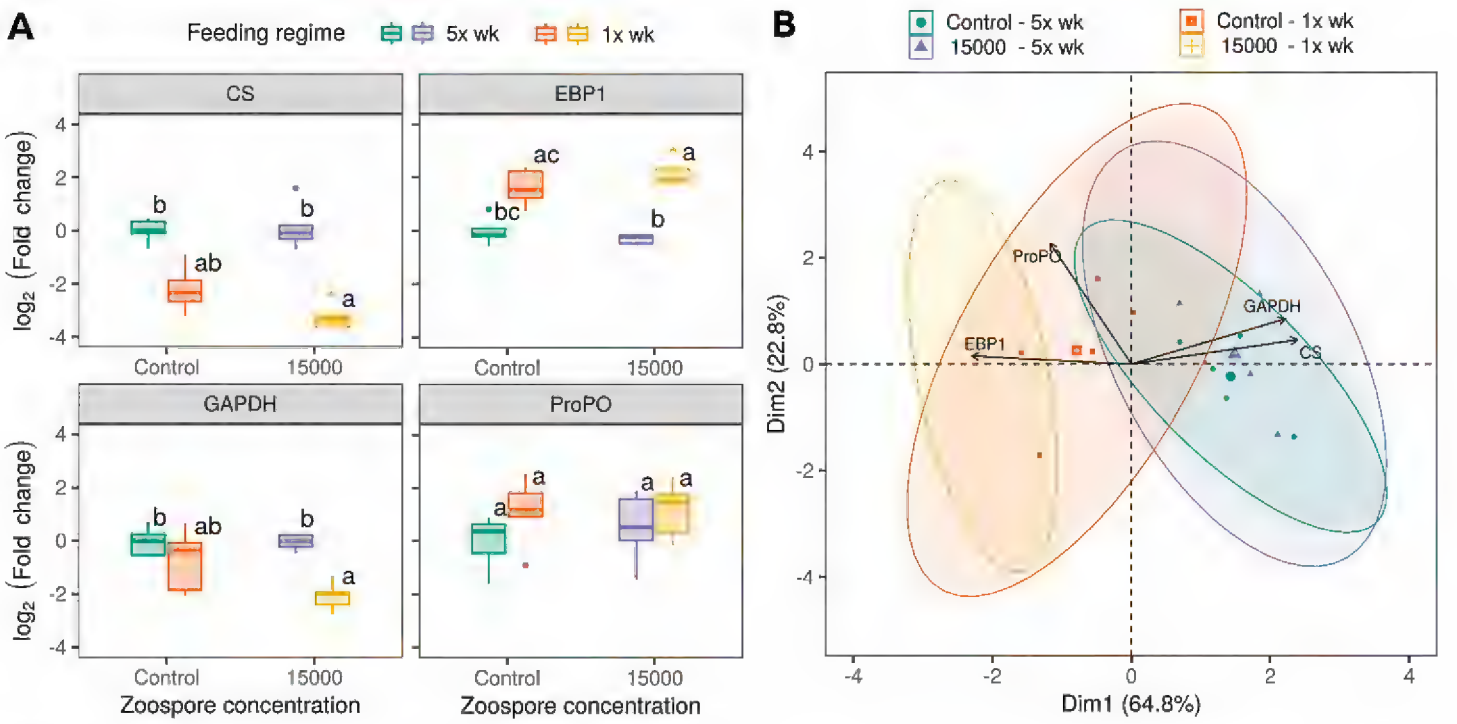

Figure 4. Differential expression of immune and metabolic genes in Experiment 2 analysed using **A** inferential comparison between groups and **B** principal component analysis (PCA). Different letters in panel A denote significant differences.

is strongly correlated with three of the gene expression variables CS, GAPDH and C/EBP-β, suggesting that these variables may vary together. PCA analysis also showed a high positive correlation between metabolic gene expressions, a positive correlation between immune gene expressions and negative correlations between metabolic and immune gene expressions. Consistent with the inferential analysis, experimental groups fed five times a week showed higher expression of GAPDH and CS genes.

Energy cost of infection

The food-restricted infected group of Experiment 2 had to activate and maintain immune response to the pathogen, inducing additional energy costs in contrast to the respective control (non-infected) group. Based on the average moisture content of the hepatopancreas, crayfish had 33.357 kJ/g in reserve. The comparison of total weight change between the two groups showed a total difference of 0.025 g, corresponding to 12.07 J/day of energy reserves used for immune response maintenance in infected individuals weighing on average 0.3 g.

Discussion

Chronic infection of invasive marbled crayfish juveniles with the *A. astaci* pathogen leads to trade-offs in energy use that reduces growth. Although this is the first such finding for crayfish, this is consistent with previous research on other invertebrates (i.e. arthropods: Rantala and Roff 2005; Bascuñán-García et al. 2010; Körner et al. 2017;

molluscs: O'Connell-Milne et al. 2016) and vertebrates (Lochmiller and Deerenberg 2000; Lee 2006; van Der Most et al. 2011; Bonneaud et al. 2012), which suggest that maintenance of an active immune system is energetically demanding, and trade-offs against growth, reproduction and development following infection are frequent. However, effects on growth observed here were achieved under repeated infections with high pathogen loads, while the observed sublethal effects (reduction of growth rates) translated into lethal effects (mortality) only under extreme food limitation. This shows that the marbled crayfish is (i) highly resistant to one of the most virulent haplogroups of *A. astaci* pathogen (haplogroup B) and can act as its carrier, and (ii) requires multiple stressors occurring simultaneously to suffer increased mortality.

Growth was reduced under both pathogen concentrations in Experiment 1 and under both feeding regimes in Experiment 2. Even though not statistically significant in some scenarios, the omnipresence of effects on growth suggest that exposure to pathogens increases allocation of energy to the immune system. Alternatively, the observed slower growth of infected groups could be the consequence of suppressed food intake suggested to occur during immune challenges (Lochmiller and Deerenberg 2000; Moberg 2000). We deem this unlikely because food was entirely consumed by crayfish in our experiments, suggesting that observed effects on growth are, indeed, related to the energy allocation to immune response.

A larger effect size of repeated infections on weight was observed in both experiments and became especially evident in Experiment 2. Here, the extreme food limitation (feeding once a week) almost completely ceased growth in both control (non-infected) and infected groups. The miniscule (if any) weight increase of the control food-restricted group suggests that those individuals were very near or at the point of starvation. Additional need to fuel the immune response of infected individuals further depleted energy reserves, thus increasing the severity of starvation resulting in increased mortality of the infected group fed once a week.

Starvation, as well as the difference in severity of starvation, was reflected in the down-regulation of genes involved in the central metabolic pathways of glycolysis and citrate cycle in both infected and non-infected group fed once a week. This is in line with previous studies on freshwater species which have shown that the expression of both CS and GAPDH decrease during starvation and fasting (Tripathi and Verma 2003; Salem et al. 2007). The most pronounced reduction in metabolic gene expression was observed in the food-restricted infected group, corroborating our assumptions that immune response to a pathogen resulted in additional depletion of energy reserves.

Food limitation and the resulting starvation induced up-regulation of the C/EBP-β transcription factor, belonging to the CCAAT/enhancer-binding protein (C/EBP) family, which are involved in the regulation of the metabolism, cell growth, differentiation, death, immune and inflammatory processes (Ramji and Foka 2002; Wang et al. 2019). C/EBP-β up-regulation is crucial in the acute phase of the *A. astaci* infection in the native noble crayfish (Boštjančić et al. 2021). In our study, C/EBP-β was up-regulated in both control and infected food-restricted groups in comparison to groups fed five times a week, with the highest change in C/EBP-β expression observed in the food-restricted

infected group. This indicates that stressful conditions of both starvation and pathogen infection increase the activity of C/EBP-β gene, signifying its role in regulation of both metabolism and immune response in crayfish. The expression of the ProPO gene, which belongs to the core immune response mechanism in crustaceans engaged in the encapsulation of the pathogen (ProPO pathway; Cerenius et al. 2003), did not differ significantly between infected and non-infected individuals in any of the groups in Experiment 2. This is consistent with previous reports from infection trials that applied a single infection using lower pathogen concentration of the same *A. astaci* haplogroup (Boštjančić et al. 2021), suggesting that there was no acute immune response and that energy was not invested into synthesis of immune effectors in hepatocytes. Rather, it probably went into the reparation of the damaged epicuticle, as the first line in the defence against the pathogen (Cerenius et al. 2003), which is energetically costly.

Lower mortality in the group fed five times a week indicates that these individuals were more able to cover the additional energy costs of fighting the disease, including the potential reparation of damage, as opposed to the group fed once a week. The scale of the cost is well represented by the differences in weight gain between the infected and non-infected food-restricted groups: the 'missing' weight can be solely attributed to energy costs of fighting the infection (immune response maintenance costs) and related consequences.

Chronic exposure to the *A. astaci* pathogen is unlikely to have a long-term effect on marbled crayfish populations, except under extreme limitation of food availability, which was used in this study as a proxy for density-dependent effects. In all experimental groups, infected individuals grew more slowly than non-infected. In nature, the slower growth could translate into slower maturation rates and/or lower fecundity of infected individuals due to size-dependence of these traits (Hossain et al. 2019) and reduction in energy available for reproduction. However, we do not expect that such growth reduction would have high implications for the invasion success of this species due to its parthenogenetic mode of reproduction and high fecundity in comparison to other native and invasive crayfish (Hossain et al. 2018).

Furthermore, a lower proportion (40 - 55%) of individuals tested positive for *A. astaci* in Experiment 1 (analyses performed six weeks after last infection) compared to Experiment 2 (analyses performed two weeks after the last infection), where almost all individuals (87 - 100%) were *A. astaci* positive. This either means that: i) infections were less successful in Experiment 1 or that ii) some individuals from Experiment 1 were able to contain the infection during the six weeks since the last *A. astaci* infection, unlike in Experiment 2 where *A. astaci* detection was performed two weeks after the last infection. We consider the former unlikely, as the procedures were the same in both experimental trials and number of infections was higher in Experiment 1 (6 repeated infections in Experiment 1 vs. 5 in Experiment 2). If the latter is true, this suggests that at least some proportion of marbled crayfish that survived repeated infections could potentially efficiently contain the pathogen and minimise further trade-offs with growth.

Our results add to the growing knowledge regarding the high tolerance of marbled crayfish to multiple single and combined stressors (Kaldre et al. 2015; Guo et al.

2019; Hossain et al. 2021; Stara et al. 2021). Such tolerance, in combination with fast growth, high fecundity and parthenogenetic mode of reproduction, prime it for becoming one of the most successful crayfish invaders.

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Supplementary material 1

Protocols, primers, statistical support data, images

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Explanation note: The file contains addtional detailed descriptions of used protocols and applied statistical analyses, additional statistical support data and images.

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